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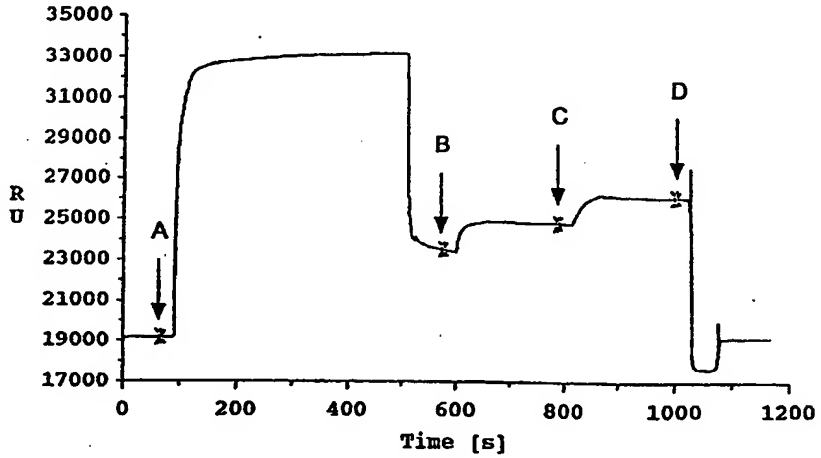

(57) Abstract

A method of assaying for at least two different analytes in a fluid sample, wherein each analyte is determined by detecting or measuring a mass change at a solid sensing surface caused directly or indirectly by the analyte, comprises the steps of co-immobilizing to the same sensing surface different catching molecules each capable of specifically binding to either a respective analyte or a respective analyte analogue or analyte specific binding partner added to the sample, and either (i) after contacting the surface with the sample, determining the binding of each different analyte, analyte analogue or specific binding partner to the respective catching molecule by sequentially contacting the obtained surface with respective specific reagents to said analytes, analyte analogues or specific binding partners, or (ii) sequentially contacting the immobilized surface with sample portions containing either different specific binding partners to the respective sample analytes or different analyte analogues to determine the binding of each specific binding partner or analyte analogue to the respective immobilized analyte or analyte analogue or specific binding partner, respectively.

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Assay for multiple analytes with CO-immobilized ligands.

The present invention relates to an improvement in assay methods of the type where the presence of an analyte is detected by contacting the sample with a solid sensing surface, and determining a mass change at the surface caused directly or indirectly by the analyte in the sample.

One class of methods for determining such changes in the mass at a sensing surface utilizes evanescent wave sensing at an optical surface. Evanescent wave sensing technology based upon surface plasmon resonance, hereinafter SPR, has recently been developed for inter alia immunoassay methods. The phenomenon of SPR is well known. In brief, SPR is observed as a dip in intensity of light reflected at a specific angle from the interface between an optically transparent material, e.g. glass, and a thin metal film, usually silver or gold, and depends on among other factors the refractive index of the medium (e.g. a sample solution) close to the metal surface. A change of refractive index at the metal surface, such as by the adsorption or binding of material thereto, will cause a corresponding shift in the angle at which SPR occurs. To couple the light to the interface such that SPR arises, two alternative arrangements are used, either a metallized diffraction grating (Wood's effect), or a metallized glass prism or a prism in optical contact with a metallized glass substrate (Kretschmann effect). For further details on SPR, reference is made to our WO 90/05295. In an SPR-based immunoassay, a ligand may be bound to the metal surface, and the interaction thereof with an analyte of an aqueous sample in contact with the surface is monitored.

In our above-mentioned WO 90/05295 and our WO 90/05305 there are described an SPR-based biosensor system and a sensor unit, respectively, permitting simultaneous or sequential measurement of several different analytes by providing several sensing or detection surface areas defined on the metal surface, each sensing area supporting a different ligand and enclosed in a respective flow cell.

The separate flow cells are arranged to be passed by the sample flow either in parallel or in series.

Similarly, US-A-4,889,427 discloses a method and apparatus for the simultaneous or sequential measurement of several different analytes by providing several separate metal strips arranged in a single flow cell to be passed by the sample flow, each metal strip supporting a different ligand, such as an antibody.

The optical and mechanical design of the systems required for performing such measurements is relatively complex. The present invention therefore seeks to reduce the complexity of such systems for simultaneous determination of two or more analytes.

In accordance with the present invention this is accomplished by co-immobilizing the different specific ligands or reagents in a single surface area and, after contacting the sample with the surface area, directly or indirectly detecting the different analytes sequentially. Co-immobilization in the present context means that these different specific ligands or reagents are randomly or orderly immobilized to the single surface area.

The present inventive concept makes use of the fact that the specific response for surface concentration, or mass, detecting devices, such as surface plasmon resonance detection, is in principle independent of the size of the detecting area, at least down to the diffraction limitations of the optical system. This is in contrast to absolute measuring devices such as used in ordinary solid phase assays where the specific response is area dependent. This independence of area size for the surface concentration detecting device will thus readily allow reduction in the number of detection areas by co-immobilization as proposed above.

As is readily understood, such co-immobilization permitting the determination of two or more different analytes in a single sensing area will greatly reduce the complexity of the optical and mechanical, including the microfluidic design of the analytical system.

The present invention therefore provides a method of qualitatively or quantitatively assaying for at least two different analytes in a fluid sample, wherein each analyte is determined by detecting or measuring a mass change at a solid sensing surface caused directly or indirectly by the analyte, which method is characterized by co-immobilizing to the same sensing surface different catching molecules each capable of specifically binding to (depending on the assay principle) either a respective analyte or a respective analyte analogue or analyte specific binding partner added to the sample, and either (i) after contacting the surface with the sample, determining the binding of each different analyte, analyte analogue or specific binding partner to the respective catching molecule by sequentially contacting the obtained surface with respective specific reagents to said analytes, analyte analogues or specific binding partners, or (ii) sequentially contacting the immobilized surface with sample portions containing either different specific binding partners to the respective sample analytes or different analyte analogues to determine the binding of each specific binding partner or analyte analogue to the respective immobilized analyte or analyte analogue or specific binding partner, respectively.

As used herein the term analyte analogue means either a molecule having a similar binding reactivity against an immobilized ligand or capturing molecule as the analyte, or the analyte conjugated with another molecule which does not change the binding characteristics of the analyte.

The term specific binding partner as used herein means a molecule which reacts specifically with a particular molecule, such as an analyte or immobilized capturing molecule.

The term specific reagent is used herein to denote a reagent used to generate the specific analyte detection signal which correlates to the analyte concentration in the sample.

The method of the invention readily permits simultaneous measurement of two different analytes, but three or even more analytes may be determined without affecting the precision of the concentration determination.

The immobilized capturing molecules may be low molecular weight (LMW) as well as high molecular weight (HMW) molecules or a mix of LMW and HMW molecules.

The co-immobilization concept is, as appears from the above definition of the method of the invention, compatible with several different per se conventional assay formats or principles for concentration determination, such as e.g. sandwich assay, inhibition assay, displacement assay, competitive assay or combinations thereof.

For instance, a sandwich assay may be performed by immobilizing different capturing molecules, such as antibodies, to the sensing surface which are capable of reacting with the respective analytes. Each analyte must in this case be bifunctional, i.e. exhibit a second binding site in addition to that binding to the corresponding immobilized capturing molecule. Reagents which are each capable of binding specifically to a respective analyte, such as antibodies, are then added in sequence, and the binding of one reagent to its respective analyte is then determined before the next reagent is added. The amount of reagent bound is proportional to the concentration of the corresponding analyte in the sample.

A competitive assay may be performed by adding analyte analogues to the sample and have the analytes compete with the analyte analogues for the binding to respective capturing molecules co-immobilized to the surface. The amount of each analyte analogue bound to the surface is then determined by sequentially contacting the surface with reagents, such as antibodies, specifically binding to the respective analyte analogues. The determined amount of analyte analogue is inversely proportional to the concentration of the corresponding analyte in the sample. The analyte analogue may, for example, be a HMW molecule such as an antibody, but may also be a LMW molecule

conjugated to a LMW or HMW molecule, such as e.g. bovine serum albumin, to which the specific reagent is directed.

In the case of an inhibition assay, analytes or analyte analogues may be co-immobilized on the sensing surface. Specific binding partners to the analytes are then added to the sample, and after contacting the sample with the sensing surface, the bound specific binding partners are determined by sequentially contacting the surface with specific reagents capable of specifically reacting with the binding partners. The latter may, for example, be antibodies conjugated with respective different molecules against which the specific reagents may be directed. As is readily understood, the determined amount of each specific binding partner is inversely proportional to the concentration of the respective analyte in the sample.

Alternatively, specific binding partners for the respective analytes may be added to separate portions of the sample containing the analytes. By sequentially contacting the different sample portions with the surface having analytes or analyte analogues co-immobilized thereto, the binding of each specific binding partner may be determined as inversely indicating the concentration of the corresponding analyte in the sample.

A displacement assay may be performed by co-immobilizing analytes or analyte analogues on the sensing surface and saturating substantially all immobilized analytes or analyte analogues by binding specific binding partners thereto. The surface is then contacted with the sample, the analytes in the sample causing the specific binding partners to be partially removed by either competing for the binding to the analytes or binding otherwise to the specific binding partners such that they are released from the surface. The amount of each remaining specific binding partner, which is inversely proportional to the respective analyte concentration in the sample, is then determined by sequential addition of specific reagents directed against the respective specific binding partners. Each specific reagent used must, of course, be directed

against another binding site on the corresponding specific binding partner than that participating in the binding to the respective surface immobilized analyte or analyte analogue.

In all the above described assay formats, the detection responses may, if desired, be increased by further sequential additions of additional specific reagents which bind to the respective species bound in the preceding detection step.

The contact between the fluid sample medium and the optical surface may be static, or preferably, dynamic, i.e. the provision of the sensing surface in some kind of flow cell.

Suitable sensing surfaces to be used in the present invention are described in our WO 90/05303 which discloses sensing surfaces capable of selective biomolecular interactions and designed to be used in biosensor systems, particularly systems based upon surface plasmon resonance (SPR). These sensing surfaces comprise a film of a free electron metal, preferably silver or gold, having one of its faces coated with a densely packed monolayer of specific organic molecules. To this monolayer a biocompatible porous matrix, e.g. a hydrogel, is bound, which matrix is employed for immobilizing suitable ligands for target biomolecules to be determined by the particular biosensor.

The mass sensing methods for which the present invention may be used are, as mentioned previously, not restricted to SPR methods, but extend to any assay method measuring a mass change at a sensing surface as being indicative of the presence of an analyte. Such methods generally include reflection optical methods, both internal and external, for example, ellipsometry and evanescent wave spectroscopy, the latter including Brewster angle reflectometry, critical-angle reflectometry, evanescent wave ellipsometry, scattered total internal reflection (STIR), optical waveguide sensors, etc.

Situations where it is of interest to analyze more than one analyte and to which the present invention thus may be applied are, for example, clinical situations requiring the analysis of more than one analyte to make a correct diagnosis or decision, and food as well as environmental analyses where it is of importance to analyse more than one analyte at a time to get a general picture of the situation. Exemplary of such clinical situations are myocardial infarction, fertility examination and transplantation surgery. The determination of antibiotics in milk is an example of food analysis, and the determination of pesticides in water may be mentioned as an example of environmental analysis.

In the following, the invention will be described in more detail by non-limiting examples, reference being made to the accompanying drawings where:

Fig. 1 is an SPR-sensor diagram showing the co-immobilization of a monoclonal antibody specific for CK-MB and a monoclonal antibody specific for myoglobin to a sensing surface;

Fig. 2 is a corresponding diagram to Fig. 1 showing the analysis of a sample containing creatine kinase MB (CK-MB) and myoglobin using the sensing surface with co-immobilized monoclonals against CK-MB and myoglobin, respectively, in Fig. 1;

Fig. 3 is a corresponding diagram to Fig. 2 showing the analysis of a sample containing ß2-microglobulin and IgE using a sensing surface with co-immobilized monoclonals against ß2-microglobulin and IgE, respectively;

Fig. 4 is a diagram showing standard curves for ß2-microglobulin obtained when analyzing ß2-microglobulin on a single-immobilized anti-ß2-microglobulin surface and on a co-immobilized anti-ß2-microglobulin/anti-IgE surface, respectively;

Fig. 5 is a diagram showing the precision profiles for ß2-microglobulin in the analysis in Fig. 4;

Fig. 6 is a diagram showing standard curves for IgE obtained when analyzing IgE on a single-immobilized anti-

IgE surface and on a co-immobilized anti-IgE/anti-β2-microglobulin surface, respectively; and

Fig. 7 is a diagram showing the precision profiles for IgE in the analysis in Fig. 6.

In the following Examples, the measurements are performed on a commercial SPR-based biosensor instrument (BIAcore™) and commercial sensing surfaces (Sensor Chip™ CM5) (both marketed by Pharmacia Biosensor AB, Uppsala, Sweden).

EXAMPLE 1

A. Co-immobilization of monoclonal antibodies on sensing surface

Immobilization on a sensing surface of a monoclonal antibody specific for CK-MB and a monoclonal antibody specific for myoglobin was performed in the biosensor instrument in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing surface was maintained at 5 µl/min. A fraction of the carboxyl groups on the sensing surface was activated to form reactive N-hydroxysuccinimide esters by injecting into the instrument 35 µl of a solution containing 0.2 M 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) in water. 35 µl of the antibody solution containing 50 µg/ml of a monoclonal antibody specific for CK-MB (obtained from BiosPacific, Emeryville, California, U.S.A.) and 50 µg/ml of a monoclonal antibody specific for myoglobin (obtained from the Institute of General and Molecular Pathology, Tartu State University, Tartu, Estonia) in 10 mM sodium acetate, pH 5.0, were then injected. A buffer with a pH below the pI of the antibody will give a positive net charge of the protein, and at low ionic strength the antibodies will preconcentrate to the remaining negatively charged carboxyl groups on the surface via electrostatic attraction giving a high antibody concentration in the matrix. The preconcentration allows fast immobilization with low amount of antibodies. Remaining reactive ester

groups were deactivated by injection of 35 µl of 1 M ethanolamine hydrochloride, pH 8.5. The sensorgram obtained is shown in Fig. 1 (response in resonance units, RU, plotted versus time in seconds). The response signal was evaluated at two levels: 20 seconds before the injection of EDC/NHS (A) and 9 minutes after the injection of ethanolamine (B). B minus A thus defines the immobilized amount of the two antibodies.

B. Analysis of plasma samples

The analysis of CK-MB and myoglobin at elevated levels in a plasma sample, using the sensing surface with co-immobilized antibodies prepared in section A above, was performed in the following manner:

A continuous flow of HBS (10 mM Hepes buffer, 0.15 M NaCl, 3.4 mM EDTA, 0.05 % Tween), pH 7.4, over the sensing surface was maintained at 5 µl/min. 35 µl of a plasma sample containing CK-MB and myoglobin were injected into the instrument. 4 µl each of second antibodies specific for CK-MB and myoglobin, respectively, at a concentration of 100 µg/ml were then injected in sequence followed by 4 µl of 10 mM glycine-HCl, pH 2.5. The sensorgram obtained is shown in Fig. 2 (response in resonance units, RU, plotted versus time in seconds). The response signal was evaluated at four levels: 20 seconds before the injection of the sample (A), 20 seconds before the injection of the second antibody specific for CK-MB (B), 20 seconds before the injection of the second antibody specific for myoglobin (C), and 20 seconds before the injection of glycine-HCl (D). Thus, A defines the baseline, B minus A defines the plasma response, C minus B defines the specific response for CK-MB, and D minus C defines the specific response for myoglobin. The analysis time was 18 minutes.

EXAMPLE 2

In the same way as described in Section A of Example 1 above, antibodies specific for β2-microglobulin and IgE, respectively, were co-immobilized to the sensing surface by injecting 15 µg/ml anti-β2-microglobulin monoclonal (Kabi Pharmacia AB, Sweden)) and 30 µg/ml anti-IgE monoclonal

(Kabi Pharmacia AB, Sweden). Analysis of β2-microglobulin and IgE was then performed by maintaining a continuous flow of HBS (see Example 1) at 5 µl/ml. 35 µl of a buffer sample containing β2-microglobulin and IgE was injected. 4 µl each of respective second antibodies to β2-microglobulin and IgE were then injected in sequence followed by 4 µl of 10 mM glycine-HCl, pH 2.5. The second antibody for β2-microglobulin was an Ig-fraction of a polyclonal antibody at 2.5 mg/ml in 10 mM Hepes, pH 7.4. The second antibody for IgE was a mix of two monoclonal antibodies at a concentration of 50 µg/ml of each in 10 mM Na-acetate, pH 5. The sensorgram obtained is shown in Fig. 3 (response in resonance units, RU, plotted versus time in seconds). The response signal was evaluated at four levels: 20 seconds before the injection of the sample (A), 20 seconds before the injection of the second antibody specific for β2-microglobulin (B), 20 seconds before the injection of the second antibody specific for IgE (C), and 20 seconds before the injection of glycine-HCl. A defines the baseline, B minus A defines the plasma response, C minus B defines the specific response for β2-microglobulin, and D minus C defines the specific response for IgE. The analysis time was 21 minutes.

EXAMPLE 3

By proceeding correspondingly as in Example 2, standard curves for β2-microglobulin analysis were obtained by analyzing β2-microglobulin standards on (i) a co-immobilized anti-β2-microglobulin/anti-IgE surface and (ii) a single-immobilized anti-β2-microglobulin surface. The standards containing β2-microglobulin or β2-microglobulin and IgE were injected in a concentration range from 0.35 to 5.55 nM over the immobilized antibody surface followed by injection of a polyclonal anti-β2-microglobulin. The second antibody responses were used to construct the standard curves. The standard curves obtained are shown in Fig. 4, and the corresponding coefficient of variation in concentration (CV) or precision profiles [(standard deviation/mean concentration) x 100] are shown in Fig. 5.

In a corresponding manner, standard curves for IgE were obtained by injecting standards containing IgE or IgE and β2-microglobulin in a concentration range from 0.35 to 5.55 nM over the immobilized antibody surface followed by injection of a mix of two monoclonal IgE antibodies. The second antibody responses were used to construct the standard curves. The standard curves obtained are shown in Fig. 6, and the corresponding precision profiles are shown in Fig. 7.

As appears from Figs. 4 to 7, the co-immobilization did not affect neither the response dynamics nor the precision in the concentration determination.

The invention is, of course, not restricted to the above specifically described embodiments, but many modifications and changes may be made without departing from the scope of the general inventive concept as defined in the subsequent claims.

CLAIMS

1. A method of assaying for at least two different analytes in a fluid sample, wherein each analyte is determined by detecting or measuring a mass change at a solid sensing surface caused directly or indirectly by the analyte, **characterized** by co-immobilizing to the same sensing surface different catching molecules each capable of specifically binding to either a respective analyte or a respective analyte analogue or analyte specific binding partner added to the sample, and either (i) after contacting the surface with the sample, determining the binding of each different analyte, analyte analogue or specific binding partner to the respective catching molecule by sequentially contacting the obtained surface with respective specific reagents to said analytes, analyte analogues or specific binding partners, or (ii) sequentially contacting the immobilized surface with sample portions containing either different specific binding partners to the respective sample analytes or different analyte analogues to determine the binding of each specific binding partner or analyte analogue to the respective immobilized analyte or analyte analogue or specific binding partner, respectively.

2. The method according to claim 1, **characterized** in that it comprises co-immobilizing to the sensing surface different capturing molecules each capable of binding to a respective analyte, contacting the sample with the immobilized surface, sequentially reacting the bound analytes with different reagents specifically reacting with the respective analytes, and determining the binding of each reagent to the respective analyte.

3. The method according to claim 1, **characterized** in that it comprises adding analyte analogues to the sample, co-immobilizing to the sensing surface different capturing molecules each capable of binding to a respective analyte

and analyte analogue, contacting the sample with the immobilized surface, sequentially reacting the bound analyte analogues with different reagents specifically reacting with the respective analyte analogues, and determining the binding of each reagent to the respective analyte analogue.

4. The method according to claim 1, **characterized** in that it comprises providing a number of samples corresponding to the number of analytes to be determined, adding to each sample a specific binding partner to a respective analyte in the sample, co-immobilizing the analytes or analyte analogues to the sensing surface, sequentially contacting the respective samples with the immobilized surface, and determining the binding of each specific binding partner to the respective immobilized analyte or analyte analogue.

5. The method according to claim 1, **characterized** in that it comprises adding specific binding partners to the respective analytes to the sample, co-immobilizing the analytes or analyte analogues to the sensing surface, contacting the sample with the immobilized surface, sequentially reacting the bound specific binding partners with respective specific reagents thereto, and determining the binding of each specific reagent to the respective specific binding partner.

6. The method according to claim 1, **characterized** in that it comprises co-immobilizing the analytes or analyte analogues to the sensing surface, reacting the immobilized analytes or analyte analogues with respective specific binding partners such that substantially no unbound analytes or analyte analogues remain on the surface, contacting the sample with the surface to partially displace bound specific binding partners therefrom, sequentially reacting the remaining specific binding partners with respective specific reagents, and determining

the binding of each reagent to the respective specific binding partner.

7. The method according to any one of claims 1 to 6, **characterized** in that said mass change is determined as a change in refractive index.

8. The method according to claim 7, **characterized** in that said change in refractive index is determined by an evanescent wave sensing method, preferably surface plasmon resonance.

9. The method according to any one of claims 1 to 8, **characterized** in that two different analytes are determined.

10. The method according to any one of claims 1 to 9, **characterized** in that the assay is performed in a flow cell.

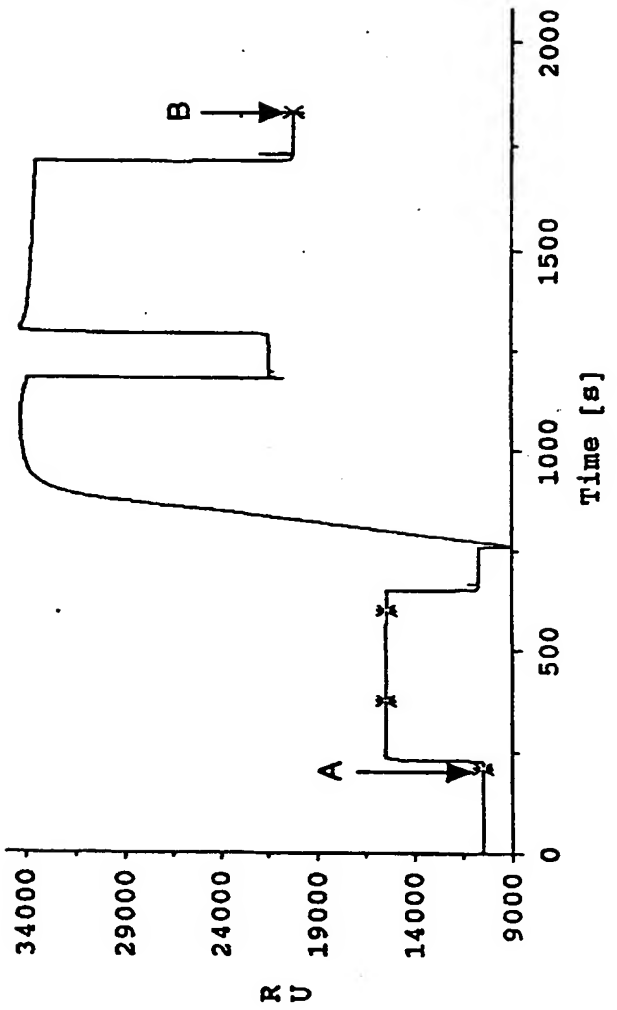
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FIG. 1

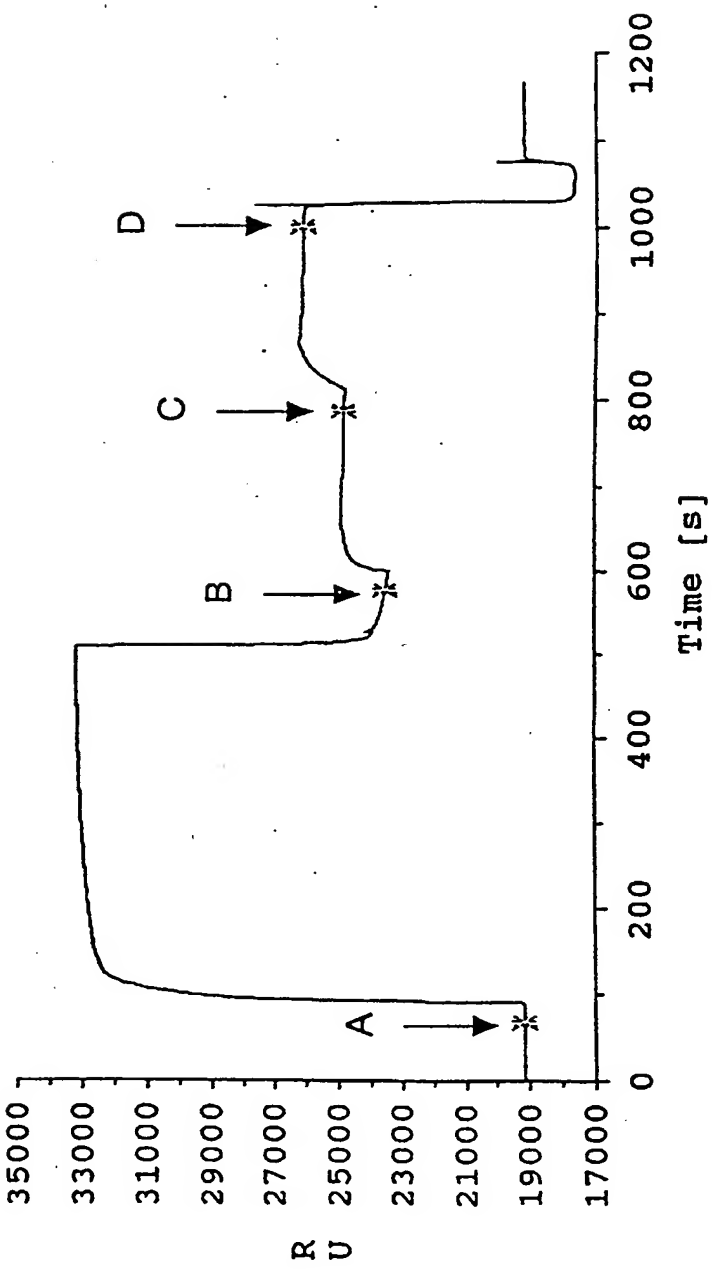
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FIG. 2

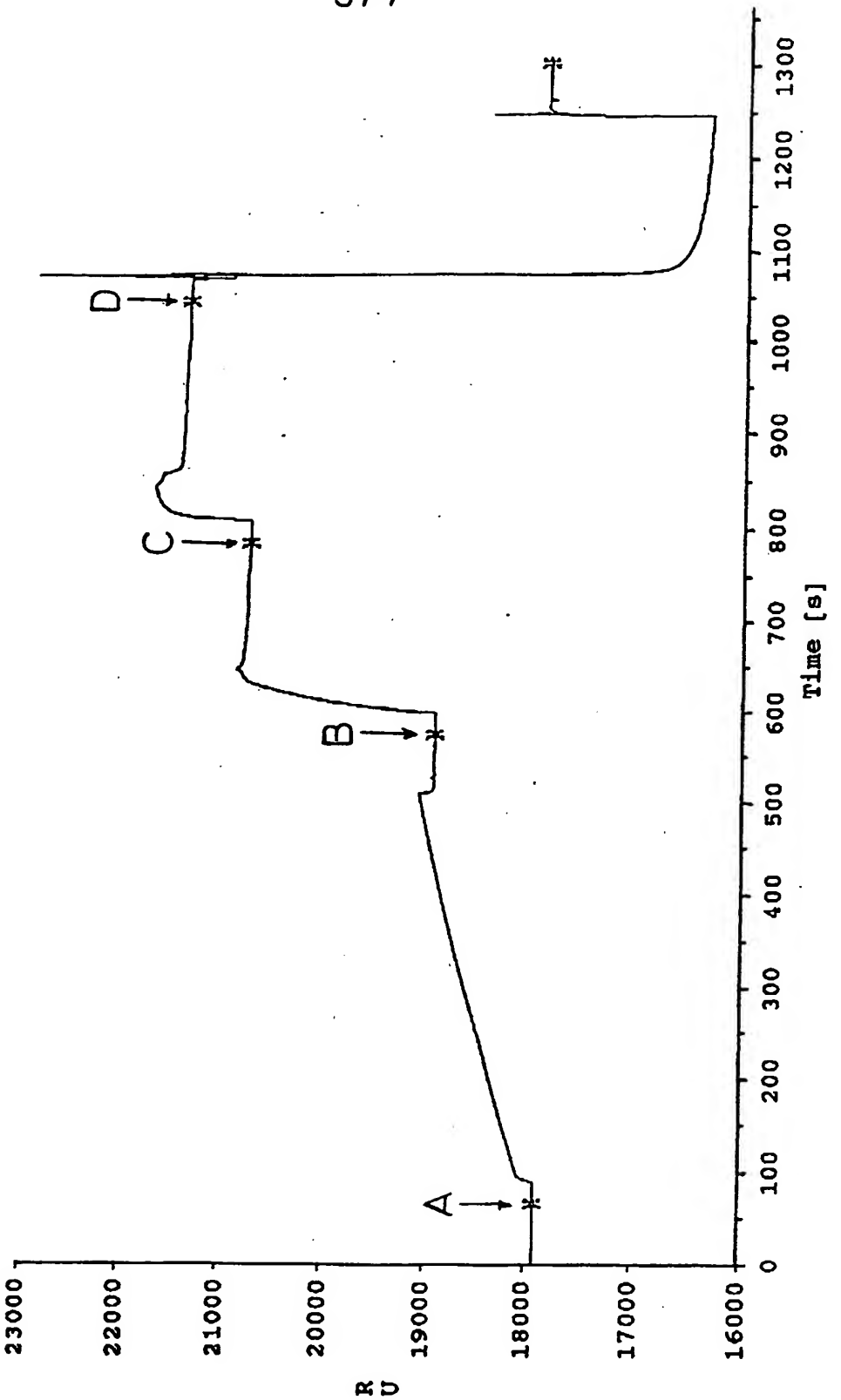
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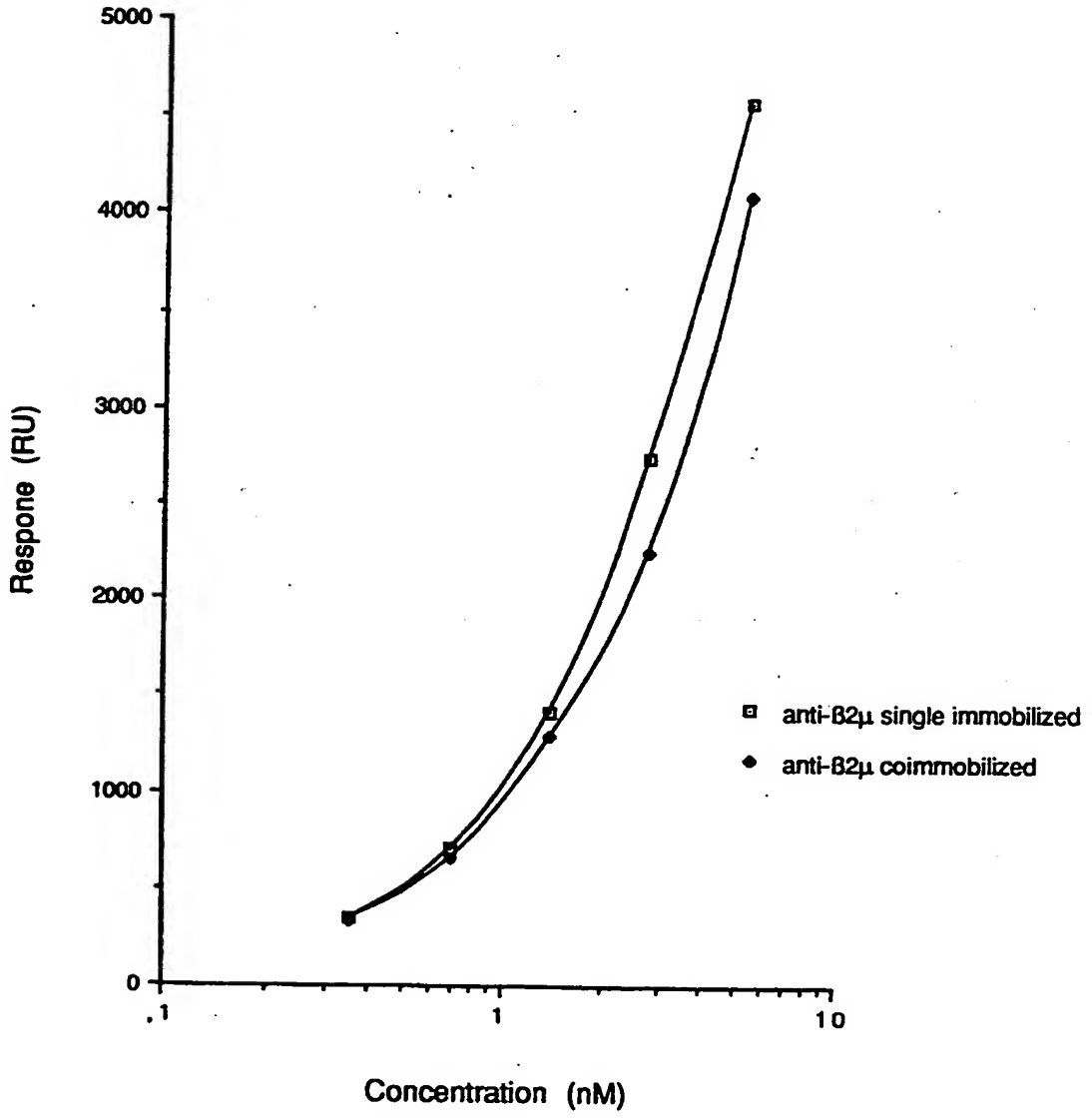
FIG. 3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4000
3000
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Concentration (nM)
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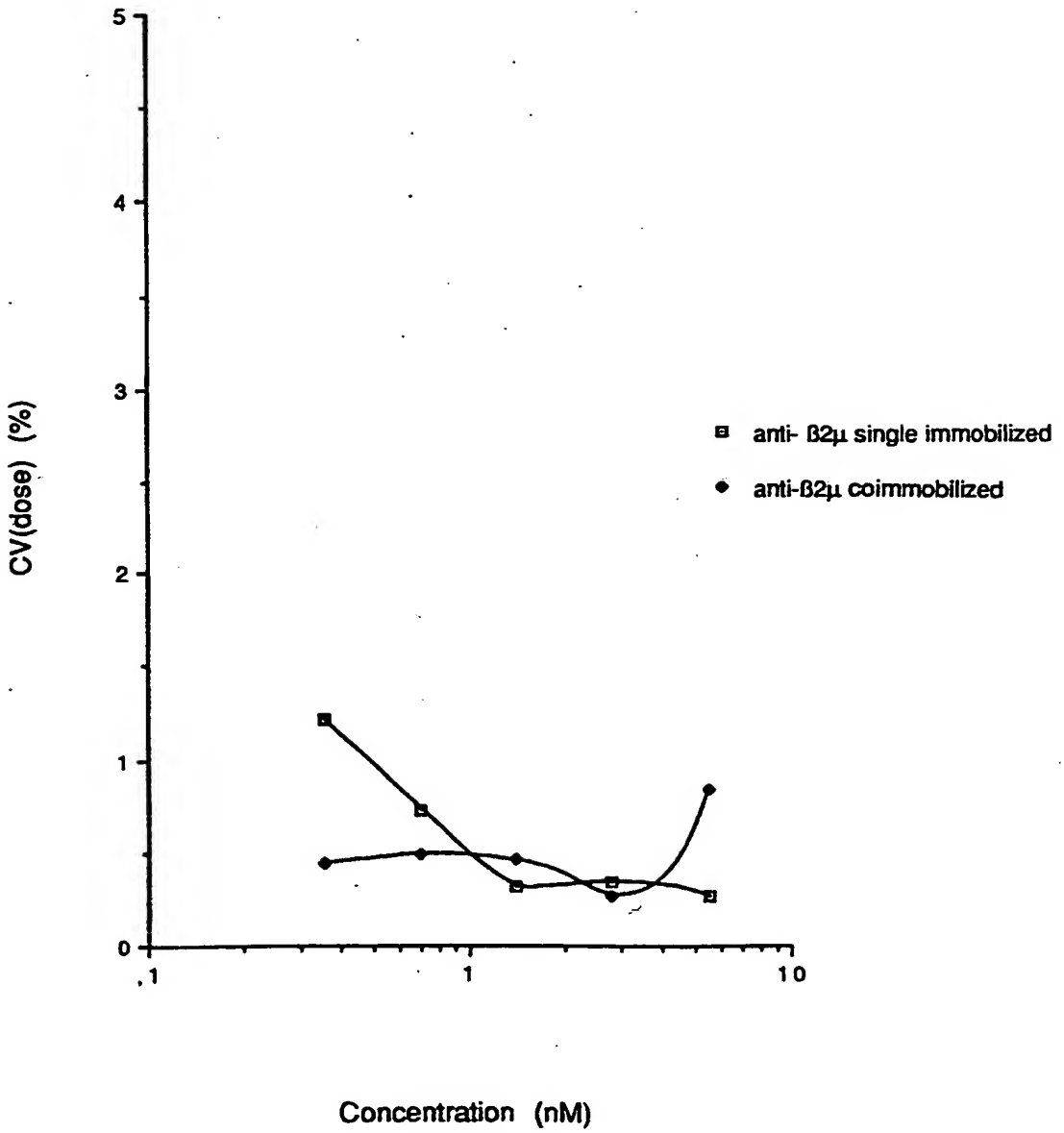
anti-β2μ coimmobilized

FIG. 4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4
3
2
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CV(dose) (%)
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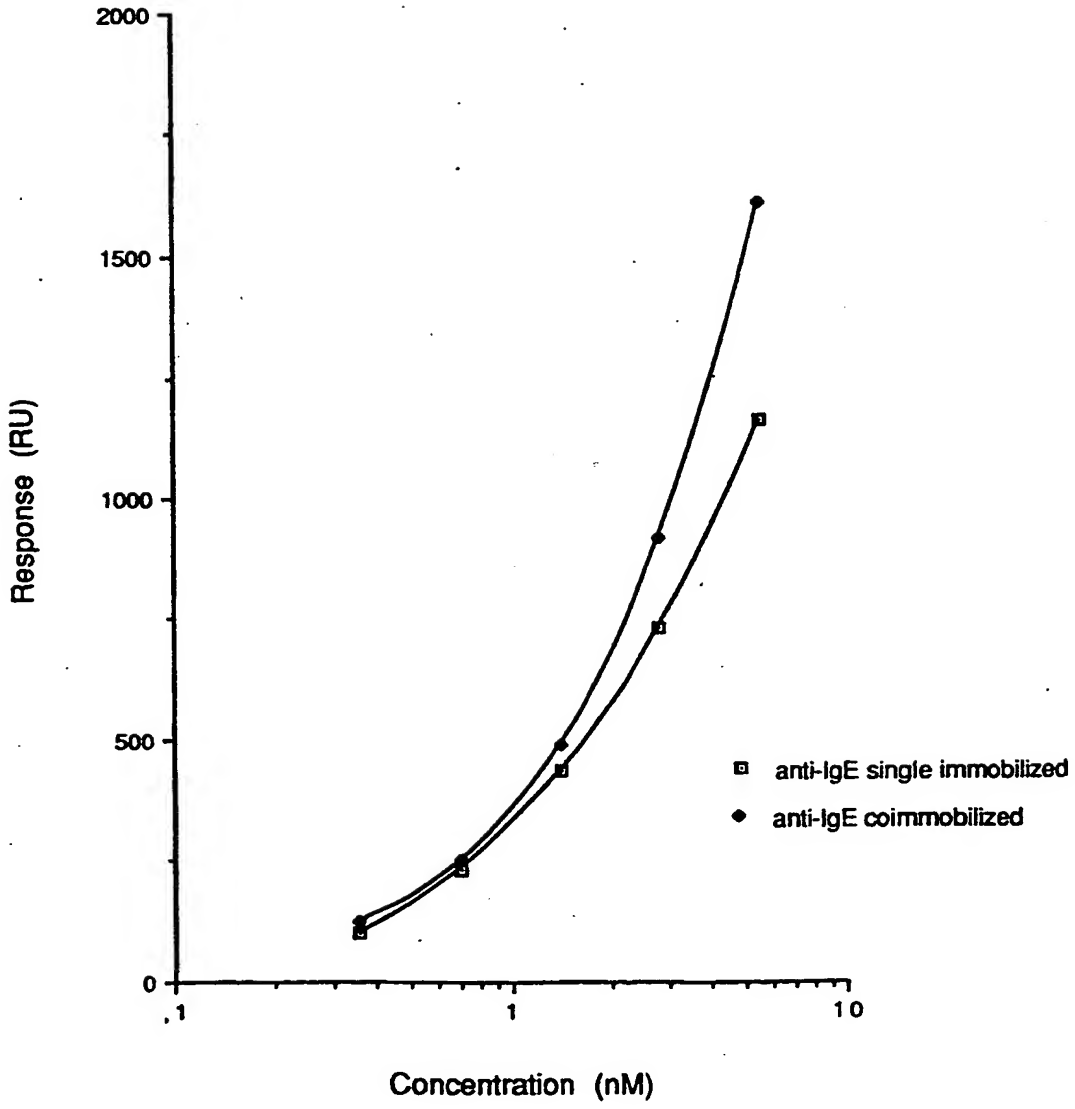
Concentration (nM)
anti- ß2µ single immobilized
anti-ß2µ coimmobilized

FIG. 5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Response (RU)
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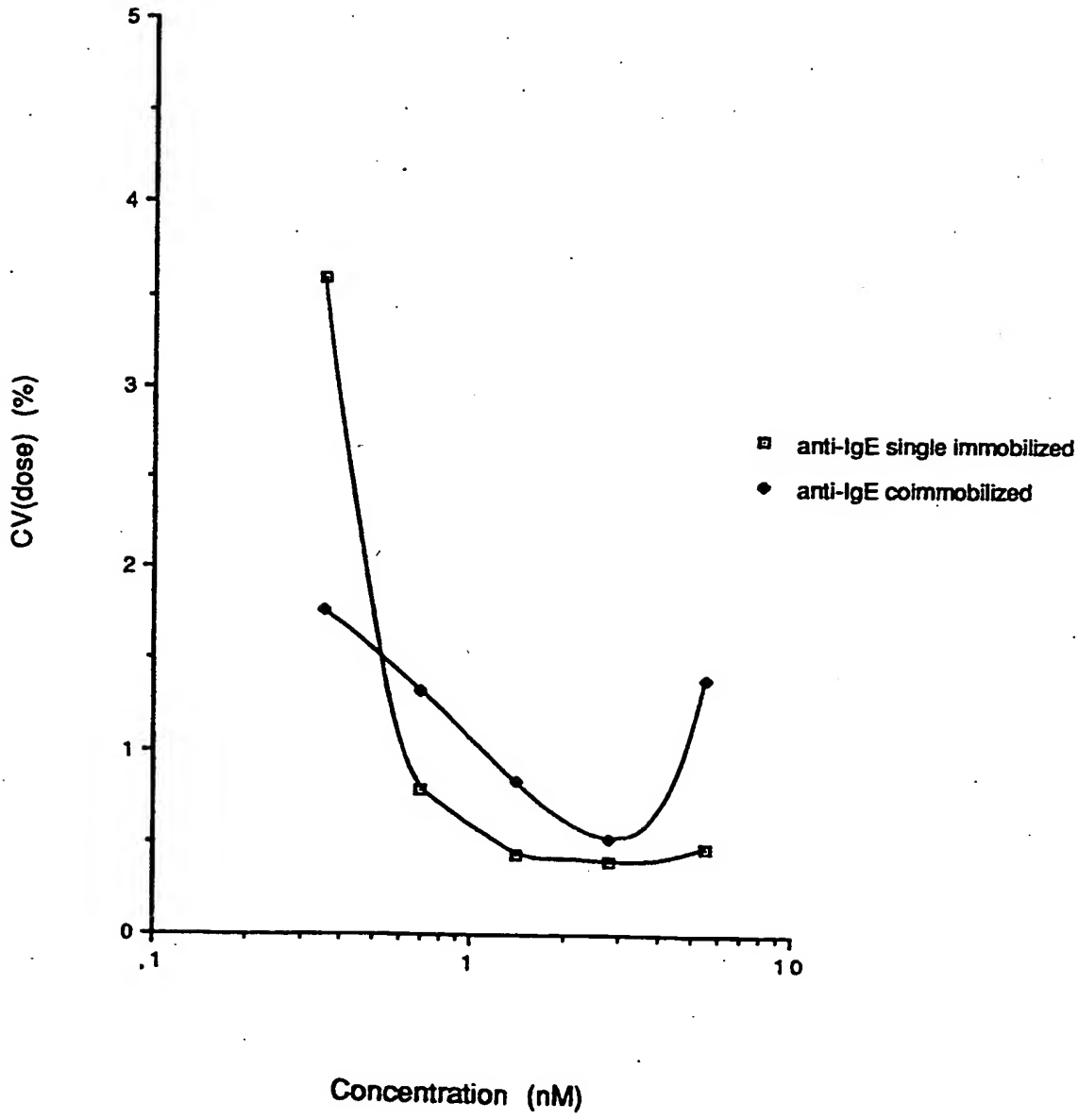
Concentration (nM)
anti-IgE single immobilized
anti-IgE coimmobilized

FIG. 6


5
4
3
2
1
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CV(dose) (%)
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1
10
Concentration (nM)
anti-IgE single immobilized
anti-IgE coimmobilized

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00488

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: G01N 33/543, C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: G01N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, CA, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO, A1, 9005306 (PHARMACIA AB), 17 May 1990 (17.05.90), page 17 - page 18"Subclass determination"	1-10
A	WO, A1, 9005303 (PHARMACIA AB), 17 May 1990 (17.05.90), page 18 III.1"Determination s of..."and Fig 3	1-10
A	WO, A1, 8002201 (MINNESOTA MINING AND MANUFACTURING COMPANY), 16 October 1980 (16.10.80), example 5	1-10

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 Sept 1993	20 -09- 1993
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00488

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

26/08/93

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